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Detection of mefenoxam-resistant strains of *Peronospora belbahrii*, the causal agent of basil downy mildew, transmitted through infected seeds

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Abstract

Epidemics of basil downy mildew (DM) incited by *Peronospora belbahrii* have been very severe in Italy since 2013, in part due to the very favorable weather conditions, and losses have occurred in many commercial farms, even after repeated mefenoxam treatments. DM populations from basil plants and seeds, which are associated with failure in downy mildew control under field and greenhouse conditions, have been tested for their sensitivity to mefenoxam. Basil plants were inoculated with a sporangial suspension of seven DM populations and treated, before and after inoculation with the pathogen, with different dosages of mefenoxam: 100 µg/ml, which corresponds to the currently applied field dosage, 200 µg/ml and 1000 µg/ml. Azoxystrobin was used at the field dosage as the chemical control. Three out of four DM populations from seeds and two out of three from basil plants were found to be able to infect basil plants in the presence of 100 µg/ml and 200 µg/ml of mefenoxam, while the field dosage of azoxystrobin (186 µg/ml) was found to be completely effective. The sensitive populations of *P. belbahrii* were completely controlled by the field dosage of both chemicals also 14 days after the last treatment. This study provides new information on the potential risk of introducing mefenoxam-resistant *P. belbahrii* inoculum in the field through seeds infected by resistant strains.

Keywords: *Ocimum basilicum*; fungicide resistance; phenylamides; downy mildew; seed-transmissions.

Introduction

Basil downy mildew (DM), which is incited by *Peronospora belbahrii* (Belbahri et al. 2005; Thines et al. 2009), is one of the most economically important basil diseases, and it has led to significant yield losses in several countries. In Europe, the disease has been reported in Switzerland, Italy, France and Belgium (Lefort et al. 2003; Coosemans 2004; Garibaldi et al. 2004a; 2005), and it has also been observed in Iran (Khateri et al. 2007), the United States, where it has been reported in several States (Roberts et al. 2009, Wick and Brazee 2009; McGrath 2010), Argentina (Ronco et al. 2009), Israel (Cohen et al. 2013) and China (Kong et al. 2015). The disease was first described as *Peronospora* sp. (Hansford 1932) in Uganda.

The rapid spread of the pathogen to all basil growing areas has probably been favoured by the fact that the pathogen is seed-transmitted (Garibaldi et al. 2004 b; Farahani-Kofoet et al. 2012), as well as by the shift of seed production to African countries, where the pathogen has been present since many years (Hansford 1932).

Several studies were conducted to better understand the etiology of basil downy mildew. Elad et al. (2016) discovered oospores in the symptomatic basil leaves and showed as high temperature apparently did not affect the pathogen survival. However, contaminated seeds are considered the primary inoculum source for basil DM because the pathogen rarely produces oospores (Cohen et al. 2013; Wyenandt et al. 2015). Garibaldi et al. (2004b) first found infected seeds by *P. belbahrii* in four out 17 commercial seed samples of basil, showing as 0.017% of infected seeds presumably resulted enough for the introduction and spread of the pathogen into areas where it has not been previously reported. Farahani-Kofoet et al. (2012) report that sporangiophores and sporangia can be easily recovered by washing seeds in 80-90% of commercial seed lots. In addition, the possibility of *P. belbahrii* to survive for several years on seeds further complicates the situation for seed producers and farmers (Farahani-Kofoet et al. 2012). The systemic infection of symptomless basil plants and seeds of basil has also been proved using a classic PCR assay (Farahani-Kofoet et al. 2012).

Because there are no known cultivars resistant or tolerant to DM, despite active research (Wyenandt et al. 2015; Ben-Naim et al. 2015), the control of basil DM is mainly based on the application of fungicides in the field (Gullino et al. 2009; Mershaa et al. 2012; Gilardi et al. 2013; Homa et al. 2014; Wyenandt et al. 2015). Among the various chemicals registered for use on basil against DM as foliar sprays, mefenoxam, which belongs to the phenylamide family (Schwinn and Staub 1987), has been applied extensively in Italy and elsewhere since 2004, because of its excellent preventive, curative and eradicative activities (Gullino et al. 2009).

Field resistance to phenylamides has been reported on a wide range of crops in several countries, and the situation is regularly updated in the FRAC Resistance Survey List (www.frac.info). Resistance was first observed for metalaxyl, the first phenylamide fungicide developed, very shortly after its introduction onto the market (Lebeda and Schwinn 1994). Field resistance of *P. belbahrii* to phenylamides was first observed and reported in Israel in 2013 (Cohen et al., 2013), and later in Italy (Pintore et al. 2016; Garibaldi et al. 2016). Failures in the control of basil DM on farms where mefenoxam was applied for its management in northern Italy have been observed starting in 2013.

This study was carried out to document changes in sensitivity to mefenoxam of P. *belbahrii* populations obtained from basil plants and seeds, and in order to understand how such populations can spread.

Materials and methods

Downy mildew (DM) populations

Starting from 2013, several DM populations have been collected in fields and greenhouses in Piedmont and Liguria, where failure in disease control with mefenoxam had been observed. Three *P. belbahrii* populations obtained from infected plants and four populations isolated from contaminated seeds were selected and have been used in this study. Different batches of seeds were collected in order to assess the level of contamination and to explain the difficulties encountered in DM management.

Two populations of *P. belbahrii* from organically produced basil grown in Piedmont were used as reference populations. A list of the tested *P. belbahrii* populations is reported in Table 1. The different DM populations were maintained on artificially infected basil leaves and stored at -20°C (Lebeda and Urban 2010).

Plant material and experimental conditions

During 2014 and 2015, trials were carried out in growth chambers, where small plastic-houses (90 cm high, 50 cm wide and 70 cm long) were built and kept at temperatures ranging from 20 to 23°C and relative humidity close to 95% in order to maintain favourable environmental conditions for DM development (Garibaldi et al. 2007).

Basil seeds from the highly susceptible cultivar Italiko (Semiorto, Salerno, Italy) were used for pathogen propagation and in the *in planta* bioassays.

Plants were produced from heat-treated seeds (65°C for 10 min), in order to guarantee the absence of contamination from infected seeds yielding 20-25 plants/plots. Plastic pots (1.5-L vol., 12 x 12 cm) contained a steam disinfested (90°C for 30 minutes) mixture of white peat: perlite (80:20 v/v) mix (Turco Silvestro, Albenga, Savona) were used. Four replicates were used for each treatment (1 pot /replicate) in a completely randomized design. The experiments were repeated at least three times for each DM population, under completely controlled environmental conditions. The most representative trials are reported in this manuscript.

Artificial inoculation

The DM populations were stored at -20°C and propagated on healthy basil plants of Genovese Gigante type, obtained from cv. Italiko heat treated seeds, 7 days before starting the trials in physically separated growth chambers. Basil leaves showing intensive sporulation of the pathogen were shaken in 100 ml of sterile water containing 2 µl of Tween 20. The sporangia suspension was filtered and diluted to a final concentration that ranged from 6.7 x 10^5 to 1x10^6 sporangia/ml. The artificial inoculation was carried out through nebulisation using a laboratory spray bottle (10 ml capacity). One ml of suspension was used for each replicate (1ml for each of the 4 pots), 24 h before or after the treatment, according to the protocol reported in Tables 2 and 3.

Products and treatment application

Three different dosages of mefenoxam (Ridomil Gold SL Syngenta Crop Protection 43,88% a.i.) were applied on the dates reported in Tables 2 and 3:100 µg /ml, which corresponds to the currently applied field dosage, and which was applied according to the manufacturer's instructions, a double dosage (200 µg /ml) and a ten times higher dosage (1000 µg /ml) compared to the field rate. Two treatments were applied at intervals of 7 days. Artificial inoculation was made with the pathogen either 24 hours before the first treatment with mefenoxam (A) or 24 hours after the treatment (B). Azoxystrobin (Ortiva, Syngenta Crop Protection, Italy, 23.2% a. i.), which is labelled and recommended on basil in Italy, was used as a reference chemical control 24 hours before the inoculation.

The treatments were made as foliar sprays 15-30 days after sowing, at 800 L ha-^{1}, using a handheld 1-L capacity sprayer.

Data collection and analysis

The plants were monitored daily and the evaluation of the percent of infected leaves (disease incidence) and of diseased leaf area affected (disease severity) was made, starting from the appearance of the first DM symptoms, using a disease rating scale. Disease severity was examined visually and calculated using the following formula: DS= [$\sum$(n° leaves × x 0-5) / (total number of leaves recorded)] with x 0-5 corresponding to: 1 = from1 to 10% (midpoint 5 %) infected leaf area; 2 = from11 to 25% (midpoint 18%) infected leaf area; 3 = from 26 to 50% (midpoint 38%) infected leaf area; 4 = from 51 to 75% (midpoint 63%) infected leaf area; 5= from 76 to 100% (midpoint 85%) infected leaf area.

All the collected data were statistically analysed by means of univariate ANOVA, with SPSS software 22, and the means were spread according to Tukey's test ($p < 0.05$).

Results

Artificial inoculation of basil plants with the seven tested *P. belbahrii* populations led to a consistent disease level in the inoculated, untreated control plants ranging from 33.8 to 68.3 % disease incidence at 7 days after the last treatment. Population no. 27 was least aggressive, followed by no. 9, all other populations were highly aggressive (Tables 2 and 3). DM symptoms started 7 to 13 days after the artificial inoculation (data not shown). The reference population no. 27 (originating from leaves) failed to infect basil plants treated with mefenoxam at the field dosage (100 µg/ml) even 14 days after the last treatment, it is therefore considered as sensitive to mefenoxam (Table 2). Population no. 20 (from seeds) showed 13.0 % of affected leaves with a sporulating leaf area of 5 % at 7 days after the last application of mefenoxam. No significant differences were recorded by spraying mefenoxam as a preventative measure (24 hours before the artificial inoculation) and curative treatment (24 hours after the artificial inoculation) (Tables 2 and 3).

Four days after the last treatments with mefenoxam at 100 and 200 µg /ml, basil inoculated with DM populations no.22, showed 30.3% to 24.5% of affected leaves with 6.6-6.8% of sporulated leaf areas, respectively, 24 hours before and after the artificial inoculation (Table 2). Thus, this population is considered as resistant to mefenoxam. A similar resistant response to mefenoxam was observed in *P. belbahrii* populations nos.17, 18 and 19, obtained from seeds (Table 3). Disease incidence and severity of the mefenoxam-treated plants were similar to those of the untreated control. The double concentration of mefenoxam reduced disease incidence and severity of DM populations nos. 9, 17 and 18, and provided statistically similar results to those obtained with azoxystrobin, applied as a chemical control (Tables 2 and 3).

Mefenoxam, applied at a rate of 1000 µg /ml, was able to completely control all the DM populations, and thus provided the same results as azoxystrobin used as a chemical control. However, this rate of application, which is ten times the recommended dosage, resulted to be phytotoxic to the basil plants and caused light leaf necrosis.

Discussion

Most *P. belbahrii* populations tested in this study, which were obtained from infected plants and from contaminated seed lots of basil, generally showed the same aggressiveness when tested on

untreated control basil plants except for no.s 9 and 27 which provided significantly less disease. Spraying basil plants with mefenoxam at 100 and 200 µg /ml, 24 hours before or after the artificial inoculation with the pathogen, did not significantly reduce disease severity and incidence of populations no.s 17, 18, 19 and 22, which are therefore considered as resistant to mefenoxam. Population no. 27 is considered as sensitive, whereas populations no.s 9 and 20 may contain a low proportion of resistant spores. Since in biotrophic pathogens it is rather tricky to produce single sporangium strains, all tested populations must be considered as mixtures of strains with different sensitivities to fungicides. The detection limit of resistant sporangia in strain mixtures is not known for *P. belbahrii* and should be evaluated for the used bioassay procedure in future experiments. Also the definition of resistant strains may vary depending on the authors. Cohen et al. (2013) reported on a resistant *P. belbahrii* population having survived a preventive treatment of potted basil plants with 1,000 µg/ml mefenoxam, while a sensitive population did not cause any symptoms after a spray with 10 µg/ml.

Although resistance to mefenoxam in populations of *P. belbahrii* obtained from infected basil plants has already been reported in Israel (Cohen et al. 2013) and in Italy (Pintore et al. 2016; Garibaldi et al. 2016), this is the first report on resistant populations of isolates of *P. belbahrii* originating from seeds. Various methods are available to assess the sensitivity of downy mildew to fungicides (Urban and Lebeda 2006). In the present study, a bioassay with whole plants has been used comparing the sensitivity to mefenoxam of DM isolates collected in field from basil plants and DM isolates from seedling obtained from contaminated seeds.

P. belbahrii was first reported in northern Italy at the beginning of 2003 (Garibaldi et al. 2004a), and later spread to other Italian production areas (Garibaldi et al. 2004b). Since its introduction, the control of this pathogen has primarily been dependent on the application of thiram and propamocab, which have only shown moderate efficacy. In previous research, it was found that mefenoxam was the most active fungicide for DM control, and a label extension, based on directive 91/414/CE, was therefore immediately requested and obtained (Gullino et al. 2009). Most basil growers applied the manufacturers' recommended rates and generally applied mefenoxam once per crop cycle, alternating with other fungicides with different modes of action, such as azoxystrobin, which belongs to the Quinone outside inhibitor (QoI) group, mandipropamid, which belong to the carboxylic acid amide CAA group, and fluopicolide, which belongs to the benzamide group. As a general rule for other downy mildews, those chemicals should be applied preventatively or as early as possible in the disease cycle, in a limited number of sprays in order to avoid the selection of resistant strains (Gisi and Sierotzki 2008; Hermann and Gisi, 2012; MacBean 2012).

Resistance to phenylamides emerged rather quickly after their introduction in many oomycetes on vegetable crops such as *Pseudoperonospora cubensis* (Reuveni et al. 1980; Katan and Bashi 1981), *Peronospora tabacina* (Bruck et al.1982) and *Bremia lactucae* (Crute et al. 1987).

Even though mefenoxam is marketed for basil treatments in a mixture with copper oxychloride in Italy, growers have observed a reduced efficacy of this fungicide and yield losses since 2013.

Seeds are generally recognised as the main source from which *P. belbahrii* survives from season to season, because the pathogen very rarely produces oospores (Cohen et al. 2013; Wyenandt et al. 2015). This study confirms the presence of resistant field populations of DM of basil, and also provides evidence that basil seeds are a potential source of mefenoxam-resistant inoculum of *P. belbahrii*. In a previous study, Thomas et al. (2014) found isolates of *Didymella bryoniae* from two seed lots resistant to thiophanate-methyl, which is commonly used for the management of watermelon gummy stem blight. The aggressiveness showed by DM populations of isolates from seeds associated with mefenoxam resistance suggested a notable ability to compete with sensitive DM populations highlighting a high risk of spread in field. However, specific studies are needed to investigate the fitness of these isolates. Our results suggest the need for anti-resistance strategies for the management of DM, not only in the field but also for seed production as well as for seed dressing. Considering the high probability of using seed lots already infected, seed dressing with fungicides with different mode of action of mefenoxam, should represent the first preventative strategy to be consider for seed producers and farmers. However, among non-chemical treatments of basil seeds with hot air (65°C for 10 min), and thyme oil may be suggested (Gilardi et al. 2015).

Moreover, the presence of mefenoxam resistant *P. belbahrii* populations in basil production areas in Italy requires a continuous sensitivity monitoring of populations in fields as well as from seeds.

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Table 1 List of the populations and their origins from basil leaves and from contaminated seeds

Code	Origin of samples/seed company	Samples
9	Castagnole, Piedmont (Northern Italy)	Basil leaves
22	Compagnia del basilico, Liguria (Northern Italy)	Basil leaves
27	Nichelino, Piedmont (Northern Italy)	Basil leaves
17	Furia, Piedmont ((Northern Italy)	Basil Seeds
18	Semiorto, Liguria (Northern Italy)	Basil Seeds
19	SAIS, Liguria (Northern Italy)	Basil Seeds
20	Franchi sementi, Piedmont (Northern Italy)	Basil Seeds

Table 2 Disease incidence (% of infected leaves) and Disease severity (% of affected leaf area) on basil plants (cv Italiko) caused by *Peronospora belbahrii* populations obtained from basil plants 4 , 7, and 14 days after the last treatments

Treatments	Dosage a.i (µg /ml) and time of application	Disease incidence caused by DM populations n											
		22				9				27			
		Dat 4[y]		Dat7		Dat 4		Dat7		Dat 4		Dat7		Dat 14	
Non inoculated control	-	0.0	a[z]	0.0	a	0.1	a	0.0	a	0.0	a	0.0	a	1.3	a
Untreated control	-	53.8	c	64.8	c	36.1	d	45.3	c	16.4	B	33.8	b	36.2	b
Mefenoxam	100 A[x]	30.3	bc	35.7	b	16.8	c	24.7	b	0.0	a	0.0	a	0.0	a
Mefenoxam	200 A	24.5	b	34.9	b	0.0	a	0.0	a	0.0	a	0.0	a	00	a
Mefenoxam	1000 A	0.0	a	0.0	a	0.0	a	0.0	a	0.0	a	0.0	a	00	a
Mefenoxam	100 B	30.5	bc	47.1	bc	13.3	bc	33.0	bc	0.0	a	0.0	a	00	a
Azoxystrobin	186 A	0.0	a	0.0	a	0.0	a	0.0	a	0.0	a	0.0	a	00	a
Treatments	Dosage a.i. (µg /ml) and time of application	Disease severity (0-100) caused by DM populations n											
		22				9				27			
		Dat 4		Dat7		Dat 4		Dat7		Dat 4		Dat7		Dat 14	
Non inoculated control	-	0.0	a	0.0	a	0.0	a	0.0	a	0.0	a	0.0	a	1.0	a
Untreated control	-	23.4	b	24.0	c	9.9	b	14.3	b	3.0	b	5.0	b	6.1	b
Mefenoxam	100 A	6.4	a	11.4	b	4.3	ab	8.9	b	0.0	a	0.0	a	0.0	a
Mefenoxam	200 A	6.8	a	8.3	ab	0.0	a	0.0	a	0.0	a	0.0	a	0.0	a
Mefenoxam	1000 A	0.0	a	0.0	a	0.0	a	0.0	a	0.0	a	0.0	a	0.0	a
Mefenoxam	100 B	9.1	a	12.7	b	6.1	b	9.9	b	0.0	a	0.0	a	0.0	a
Azoxystrobin	186 A	0.0	a	0.0	a	0.0	a	0.0	a	0.0	a	0.0	a	0.0	a

[x] Time of application: A, 24h before the artificial inoculation of the pathogen. B, 24h after the artificial inoculation of the pathogen

[y] Days after the last treatment

[z] Values with the same letter in the same column are not significantly different, according to Tukey's Test ($p<0.05$)

Table 3 Disease incidence (% of infected leaves) and Disease severity (% of affected leaf area) on basil plants (cv Italiko) caused by *Peronospora belbahrii* populations obtained from basil seeds 4 and 7 days after the last treatments

Treatments	Dosage a.i. (µg /ml) and time of application	Disease incidence caused by DM populations n							
		17		18		19		20	
		Dat 4[y]	Dat7	Dat 4	Dat7	Dat 4	Dat7	Dat 4	Dat7
Non inoculated control	-	0.0 a[z]	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
Untreated control	-	54.7 c	68.3 c	55.1 c	61.7 c	51.7 bc	60.0 b	46.0 b	54.3 b
Mefenoxam	100 A[x]	23.1 b	38.5 b	33.2 bc	47.0 bc	57.9 c	64.2 b	5.3 a	13.0 a
Mefenoxam	200 A	0.0 a	18.7 ab	18.4 ab	27.1 ab	40.6 b	52.4 b	0.0 a	0.0 a
Mefenoxam	1000 A	0.0 a	0.0 a	0.0 a	2.3 a	0.0 a	0.0 a	0.0 a	0.0 a
Mefenoxam	100 B[b]	18.5 ab	37.1 b	34.2 bc	39.1 bc	40.3 b	51.1 b	0.0 a	0.0 a
Azoxystrobin	186 A	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
Treatments	Dosage a.i. (µg /ml) and time of application	Disease severity (0-100) caused by DM populations n							
		17		8		19		20	
		Dat 4	Dat7	Dat 4	Dat7	Dat 4	Dat7	Dat 4	Dat7
Non inoculated control	-	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
Untreated control	-	23.0 b	34.6 d	20.5 b	26.8 b	17.8 cd	22.7 c	24.3 b	27.2 b
Mefenoxam	100 A	4.8 a	12.9 bc	9.2 ab	13.5 ab	18.7 d	20.8 c	3.0 a	5.0 a
Mefenoxam	200 A	0.0 a	2.8 ab	3.2 a	6.8 a	12.6 bc	18.7 bc	0.0 a	0.0 a
Mefenoxam	1000 A	0.0 a	0.0 a	0.0 a	0.5 a	0.0 a	0.0 a	0.0 a	0.0 a
Mefenoxam	100 B	4.4 a	15.1 c	8.3 a	12.8 ab	8.3 b	13.0 b	0.0 a	0.0 a
Azoxystrobin	186 A	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a

[x] Time of application: A, 24h before the artificial inoculation of the pathogen. B, 24h after the artificial inoculation of the pathogen

[y] Days after the last treatment

[z] Values with the same letter in the same column are not significantly different, according to Tukey's Test ($p<0.05$)